# The thrombin potential

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# The Thrombin Potential

## A Parameter to Assess the Effect of Antithrombotic Drugs on Thrombin Generation

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**Key words:** Heparin determination – Thrombin potential – Anti-factor IIa – Anti-factor Xa, APTT

## Summary

On basis of the present knowledge and experience one can not select a laboratory test that represents the antithrombotic effect of heparin in a patient. It is shown that on basis of the existing evidence neither the APTT, the anti-factor Xa test nor the anti-factor IIa test can be accepted as such. We suggest that the surface under the thrombin generation curve measured in clotting plasma is potentially a more relevant parameter. This measurement would not only apply to treatment with heparin and heparinlike drugs of any kind but also to oral anticoagulation.

## Introduction

One of the main problems in developing a more efficient antithrombotic treatment with heparins or related drugs is to find the right laboratory correlate to the antithrombotic action in the patient. It would be extremely useful if there would exist a test that can be carried out on the plasma of a patient, and the outcome of which would correlate directly with the antithrombotic potency obtained in the patient. Such a test at this moment does not exist, so it is necessary to develop one.

Obviously it will be impossible to select this test on basis of laboratory research only, because antithrombotic efficacy is a clinical concept and cannot be translated *a priori* in laboratory terms. What we can however do is judge the merits of the available tests, select a plausible candidate and estimate its outcome in patients in which, on basis of dose finding studies, an efficient antithrombotic therapy has been installed. Then one can investigate whether identical values are obtained in equally well anticoagulated patients. Even though one cannot decide in favour of a specific test on basis of laboratory research only, it is already possible to falsify existing claims on basis of our

knowledge of the mechanism of action of heparins and heparinlike drugs. The prothrombin time e.g. would obviously not do.

In this article we will explain why we think that the current tests of heparin acitivity do not suitably reflect the effects of heparin and we will propose the outlines of a new one.

One general assumption underlies the use of both our new suggestion and the old tests viz: that antithrombotic drugs excert their action via their influence on the clotting mechanism. In view of the observation that the two main classes of antithrombotics, i.e. oral anticoagulants and heparins have nothing in common but the sole fact that they influence the amount of free thrombin formed in clotting plasma, this assumption seems not unreasonable.

In view of the importance of thrombin, we will start with a brief reminder of the mechanism of thrombin generation and the influence of antithrombotics on it.

## 1. The effect of antithrombotics on thrombin formation

On the basis of recent research it is possible to sketch a plausible picture of the course of events that leads to blood coagulation and the ways in which heparin and related drugs inhibit this process. Central to the comprehension of the inhibition is the concept of positive feedback. This mechanism as such is known for more then 40 years (1, 2, 3), yet its importance for the understanding of the mode of action of heparins seems to have been somewhat neglected. Feedback activation means that the product of the reaction, i.e. *thrombin* activates some of the reactions that lead to its own formation. This implies that in order for the mechanism of coagulation to be operative, thrombin has to be formed first by another mechanism. We therefore cannot speak of *the* mechanism of blood coagulation. There must exist several successive mechanisms of blood coagulation. The best known is the last one, i.e. the full-blown mechanism that is responsible for the explosive thrombin formation occuring after a certain lag phase. It should always be kept in mind however that in order to obtain the first traces of thrombin, during the lag-phase a partial mechanism must be operative that allows enough thrombin to be formed for the full mechanism to get going.

### 1. The full coagulation mechanism

Described in detail elsewhere (4), the full coagulation mechanism will be exposed here only in shorthand. Prothrombin is activated by the prothrombinase complex that consists of factor Xa and factor Va adsorbed next to each other on a surface constituted by negatively charged phospholipids. Factor Xa is formed

by the action of either the extrinsic- or the intrinsic tenase complex. The extrinsic tenase consists of a lipoprotein, called tissue factor (TF), to which factor VII(a) is adsorbed. The intrinsic tenase is a negatively charged phospholipid surface to which factor IXa and factor VIIIa are adsorbed. The extrinsic tenase is not only capable of activating factor X but can also activate factor IX. It can thus contribute to the formation of intrinsic tenase. In this way a reinforcement loop is formed: the Josso loop.

The alternative way to activate factor IX is via the contact activation pathway; that is by factor XIa, resulting from the interaction of a negatively charged (glass-, kaolin-, ellagic acid-) surface with the factors XII, XI, prekallikrein and high molecular weight kininogen. The physiologic importance of this pathway remains questionable. It is however the mechanism operative in the activated partial thromboplastin time (APTT).

## 2. Partial coagulation mechanisms

The full coagulation mechanism, that serves to produce the bulk of thrombin in clotting plasma – and presumably at the sites of haemostasis and thrombosis – cannot be operative from zero time on, for the very simple reason that blood immediately after the reaction has been triggered does not contain three of its main reactants to wit: Factor VIIIa, factor Va and the bulk of procoagulant phospholipids. These are produced only *after* the first traces of thrombin have been formed. In order to start full-blown thrombin formation, *incomplete, partial* mechanisms have to form small amounts of thrombin first.

One of the possible incomplete mechanisms and for the purpose of this presentation the only one to be discussed, is the one that might be operative in blood that comes into contact with a damaged cell (Fig. 1).

Fig. 1. The Lag-Time-Phase

| Enzyme | Substrate | | Product |
|---|---|---|---|
| Tiss. Thrpl + VII(a) | X | → | Xa |
| | IX | → | IXa |
| IXa + PL | X | → | Xa |
| Xa + PL | II | → | IIa |

There, tissue thromboplastin will combine with factor VII and form an – inefficient – extrinsic tenase. This forms slowly some factor IXa and some factor Xa. These factors activate factor VII so that the extrinsic tenase becomes more efficient. The factor IXa may form some more factor Xa if small amounts of procoagulant phospholipid escape from wounded cells. With the same phospholipid factor Xa can activate some prothrombin and in this way give rise the generation of small amounts of thrombin. This thrombin then can activate: a) factor VIII, b) factor V and c) platelets. In this way the factors VIIIa, Va and procoagulant phospholipids become

available and full thrombin formation sets in (Fig. 2). The clotting process thus can be thought to exist of three separate phases: a) partial triggering mechanism(s), b) thrombin mediated feedback and c) full blown thrombin formation.

Fig. 2. The Feedback Phase

| Enzyme | Substrate | | Product |
|---|---|---|---|
| IIa: | V | → | Va |
| | VIII | → | VIIIa |
| | Platelets | → | procoag. PL. |

## 3. Clotting times and thrombin formation

Clotting invariably occurs when a level of 10 – 20 nM of thrombin is attained in blood or plasma. That is shortly after the onset of the full coagulation mechanism. The clotting time therefore measures the lag-time of thrombin formation during which mainly partial triggering mechanism and thrombin mediated feedback are operative (phases a and b). Consequently measuring the influence of a drug such as heparin on a clotting time essentially measures its effect on a partial coagulation mechanism and not on the full mechanism.

A simple clinical fact illustrates that the lag time of thrombin formation – and therefore the clotting time – cannot be the only parameter from which to judge antithrombotic action. A patient with a congenital antithrombin III deficiency of 50% will have an important thrombotic tendency. Yet none of the possible variants of the clotting time will shown significant prolongation. The parameter that *is* influenced is the *amount* of thrombin formed during the action of the full coagulation mechanism. The idea that the amount of thrombin formed in a patient is related to his thrombotic tendency is supported by more clinical observations. The fact that all efficient antithrombotic therapies influence the amount of thrombin produced but do not necessarily affect the lag time is the most important one. Low-molecular-weight (LMW) heparins for instance are efficient antithrombotics and yet hardly influence clotting times. Dermatan sulfate affects clotting times even less. Mind that we do not say that the antithrombotic potency may not, under circumstances, be related to (a variation of) the clotting time. Oral anticoagulant treatment and the prothrombin time (P. T.) show that this is not true. The fact however that efficient antithrombotics exist that do not influence a clotting time, whereas these drugs all influence the amount of thrombin formed is the crucial observation.

## 2. Heparin measurement

Heparin, both in its unfractionated and in its low molecular weight forms, can be demonstrated on basis of one or more of the effects caused by its potentiation of antithrombin III (AT III). There are three main forms of test: the activated partial thromboplastin time (APTT), the anti-factor

IIa (anti-thrombin) test and the anti-factor Xa test. The prolongation of the thrombin time is a form of antithrombin test. The Heptest is a hybrid form of an APTT and an anti-Xa test.

The APTT comes nearest to an overall test of the coagulation mechanism. It measures essentially the influence of heparin on the partial clotting mechanisms that are operative when thrombin formation is triggered via contact activation. We have demonstrated that the essential point is, that heparin lowers the available thrombin concentrations during the lag phase so that factor-VIII activation is retarded (5). Factor-IX activation is normal, however. Physiologically factors VIIIa and IXa form intrinsic tenase that is practically immune to the action of AT III-heparin. When no factor VIIIa becomes available the free factor IXa that is generated by contact activation is vulnerable to the attack by AT III-heparin. Therefore the capacity to form complete tenase will be retarded. Consequently the formation of factor Xa will be retarded and diminished. If factor Xa is formed then it may be attacked in its turn when, due to the low ambient concentration of thrombin, no factor Va is present. For unknown reasons the latter mechanism does not seem to be rate limiting. Else not only the contact activated APTT but also the tromboplastin induced PT would be heparin sensitive.

Both the anti-factor IIa and the anti-factor-Xa test measure properties that, in a well designed test, are functions of the heparin concentration only. Different functions though. It is common knowledge that, for an AT III molecule to be activated towards factor Xa, it is necessary and sufficient that its heparin binding site binds to a heparin. A pentasaccharide of defined composition is the minimal structure required (6). Larger heparins may be more efficient because of a more efficient interaction with AT III but not because of binding to factor Xa. The anti-Xa level therefore indicates the number and efficacy of the AT III binding sites in a heparin. For efficient thrombin inhibition however, it is necessary that thrombin also binds to the heparin. According to the "sliding hypothesis" binding of thrombin and AT III to the same heparin molecule enhances the inactivation of thrombin importantly (7 – 10). For this to happen it is necessary that the heparin is significantly larger then its AT III binding site. Anti-factor IIa based determinations of heparin therefore measure that subfraction of AT III binding sites that is embedded in an efficient, larger molecular weight heparin.

## 3. The laboratory correlate to heparin efficacy

As explained above the three tests that are currently in use for assessing heparin in a patient plasma measure quite different properties. It has not

been proven and it is not to be expected that one of these properties directly reflects the therapeutic efficacy of heparin. As exposed, the APTT is sensitive to the influence of heparin on the partial clotting mechanism, operative during the lag time of thrombin formation. This will not necessarily correlate with the inhibition of the bulk of thrombin formation by the full mechanism. The anti-factor Xa action is the property most frequently used for assessing heparin levels in patients, were it only because it is easily measurable with the aid of a commercialized kit and because it changes significantly after injection of LMW heparin. It has been claimed that its results correlate with the therapeutic efficacy (11 – 18). Others however have not found this relationship (19 – 26). Dose finding studies with different commercialized LMW heparins lead to doses that do not differ more then 100% as to the anti-Xa levels obtained. This is readily explained by the similarity of these products. The correlation between efficient dosis and anti-factor Xa level quickly disappears when less similar preparations are investigated. Unfractionated heparin causes low anti-factor Xa levels and the specific pentasaccharide has to be administrated (in animals) in doses causing anti-factor Xa levels that are 10 x higher then that of the other LMW heparins (26). We therefore tend to accept rather the negative evidence and question the value of anti-factor Xa measurements.

More important still is the observation that both UFH and LMW heparin do not influence the thrombin generated in clotting plasma to any significantly further extend then can be explained by their anti*thrombin* properties (5). In other words, the anti-factor Xa capacity of both UFH and most LMW heparins does not or hardly influence the generation of thrombin in plasma, at least in the extrinsic system. This is explained by the fact that factor Xa is produced in tremendous excess and therefore very difficultly will become rate limiting. As we have discussed above, in the intrinsic system the situation is different. There the fast disappearance of thrombin brought about by heparin during the lag phase causes a retardation of factor VIII activation and thus an increase of the lag phase. During this time heparin-AT III can attack factor IXa that is formed normally. When finally factor VIIIa is formed it finds only small amounts of factor IXa left to form intrinsic tenase activity. This activity is consequently diminished. In mechanisms were factor VIII plays a role the influence of a heparin on factor IXa is a consequence of anti*thrombin* action because of the fact that it is the lack of thrombin that causes no factor VIIIa to be available to protect factor IXa. So anti-factor IIa and anti-factor Xa effects will tend to reinforce each-other. An effect of anti-factor Xa activity has as yet not been demonstrated.

It is anyhow clear that anti-factor Xa activity cannot be the physiolo-

gical correlate to the antithrombotic action of heparin. Anti-factor IIa remains a better candidate but must be discarded on basis of the anti-factor IXa – anti-factor IIa synergy described above. Whether a laboratory test is a good correlate to antithrombotic action can only be decided on basis of correlations in clinical trials. Yet we think that at this moment it is more likely then not that the *amount* of thrombin formed by a clotting plasma under the influence of heparin has something to do with the antithrombotic action. Measuring thrombin production is a much more direct way of measuring the effect of heparin and once one accepts that the antithrombotic effect of heparin, like that of oral anticoagulation, is due to its effect on thrombin (formation and/or breakdown), a supposition also tacidly at the basis of the current tests, it is a very acceptable candidate for the laboratory correlate.

## 4. The thrombin potential

"After all it is more like cooking then like anything else" goes the famous saying of Rosemary Biggs, speaking of coagulation research. Indeed, in the light of what we know now, we can only admire the ways in which the clotting mechanism was forced to render its secrets by measuring clotting times in clever combinations of clotting mixtures. These results led to a correct overall scheme of coagulation. After the clotting age came the age of biochemistry. All factors were purified and the enzymology of their interactions was studied in meticulous detail. What remains to be completed is a study in physiological chemistry. Experiments on the isolated organ called "blood" are to reveal what reactions that are biochemically possible are important physiologically. Blood includes plasma and blood cells of all sorts. One might also include the endothelium and the subendothelium. Modern hemostasis research more and more reckons with these formed elements. It is strange to realize how far clinical biology lags behind the state of the art in physiological chemistry. Either it resources to the APTT, a direct descendant of "first generation" clotting tests or it measures single properties like anti-factor IIa or anti-factor Xa capacity without taking into account the obvious fact that the concentration of only one effector in a strongly non-linear network like thrombin generation will hardly account for the effects observed.

We therefore tried to find a parameter that reflects as precisely as possible the processes that are influenced by any type of anticoagulant drug. I.e. a procedure beyond the scope of the determination of an individual factor or a clotting time. We think we have found this procedure in the thrombin generation assay (T.G.A.). In a series of studies we have confirmed that indeed the T.G.A. is influenced by all classes of confirmed antithrombotic drugs (5,28 – 31). We have shown that oral anticoagulation as well as heparin and

heparinlike drugs influence this test, be it by completely different mechanisms. We also showed how, by mathematical dissection of the curve, it was possible to discern between the effects of a drug on thrombin breakdown or on prothrombin conversion (32).

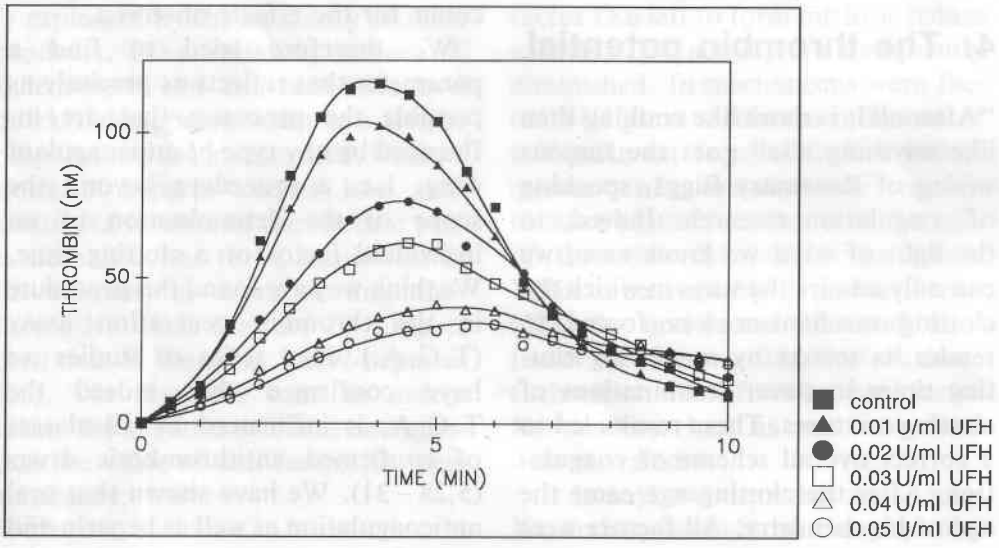
To obtain a precise thrombin generation curve is a tedious procedure not applicable to routine use in the clinical laboratory. We therefore set out to find a simple test that directly assesses one of the critical parameters of this curve. Because thrombin is an enzyme one can assume that a high concentration acting during a short time will have the same effect as a low concentration acting during a long time. The concentration-time integral, i.e. the surface under the curve may therefore conceivably be an interesting parameter to measure. In fact it is a timehonoured procedure to measure this surface to render the result of a T.G.A. when it serves for two-stage prothrombin estimation (3). This surface we will henceforward call the *thrombin potential.*

For the purpose of this article we determined the thrombinpotential by calculation from thrombin generation curves. Because the thrombin potential is a surface in the concentration-time plane of thrombin, its dimension is concentration multiplied by time. We calculated it as as nanomoles per liter times minutes. The normal value was $487 \pm 21$ nM.min (n = 12).

## 5. The influence of antithrombotics on the thrombin potential

Figure 3 shows a series of thrombin generation curves, obtained as de-

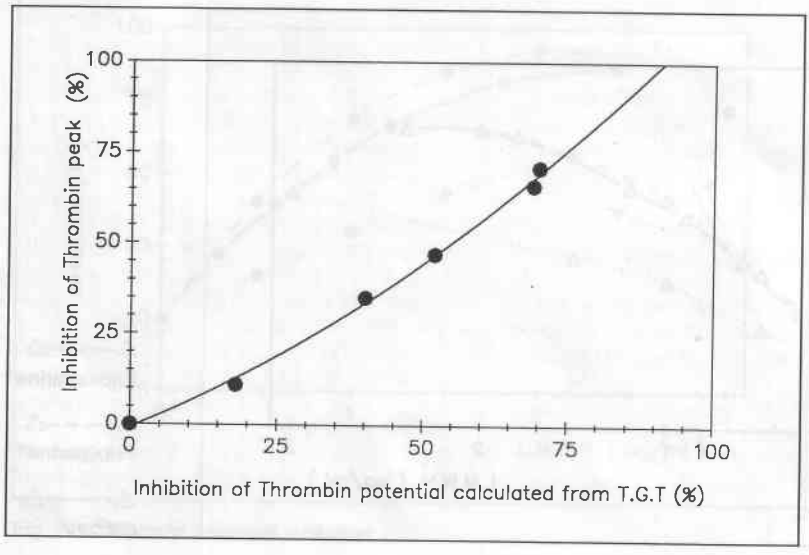
Fig. 3. The influence of UFH on thrombin generation triggered via the extrinsic system



scribed in ref. 5 and corrected for the contribution of the amidolytically active $\alpha_2$-macroglobulin-thrombin complex. From Figure 4 it is seen that the thrombin-potential follows closely the thrombin-peak. This thrombin- peak is the value that we used until now to determine the overall effect of an antithrombotic drug. Its value is strongly linked to the thrombin-potential.

The reasons to prefer the thrombin-potential are both theoretical and practical. On a theoretical basis one can, as explained above, assume that much thrombin during a relatively short time will have the same effect as less thrombin during a proportionally shorter time. It has been proven for the action of thrombin on fibrinogen that the product of thrombin concentration and clotting time is constant (33). This means that at least for that experimental system our assumption is true. It seems not far fetched to extend it to other actions of thrombin. Also a thrombin potential can be measured with more precision than a thrombin peak value. From discontinuously measured curves, as in Figure 3 one can make a reasonably exact guess of the peak values, but this needs frequent sampling and the possibility remains that the actual peak of the curve is missed by the experimental points. For the surface under the curve however, all measuring points are used and missing the peak has no serious consequences. Also, the experimental error with which the peak is measured is equal to – or larger then – the error of an individual point. The error of all the measuring points used in determining the surface will equal out as in determining a mean i.e. the error will be roughly equal to the experimental er-

Fig. 4. The relation between thrombin potential and thrombin peak in heparinized plasma



ror of a single point devided by the square root of the number of points.

The third and main point however, is that we have good hope to design a method that in one simple chromogenic test measures the thrombin potential. If indeed this test comes out as we hope it does then it will be possible to follow the thrombin integral in the clinical laboratory as a routine procedure.

As a prelude we measured the influence of different low molecular weight heparins on the thrombin integral, the thrombin peak and the prothrombinase peak (Figs. 5 – 6 and 7). We also compared the influence of three different LMW heparins on the thrombin potential (Fig. 6). In order to show that the thrombin integral indeed varies with the anticoagulant level in patients we compared the thrombin potential and the prothrombin level in a series of 23 anticoagulated patients and 4 normal subjects (Fig. 8).

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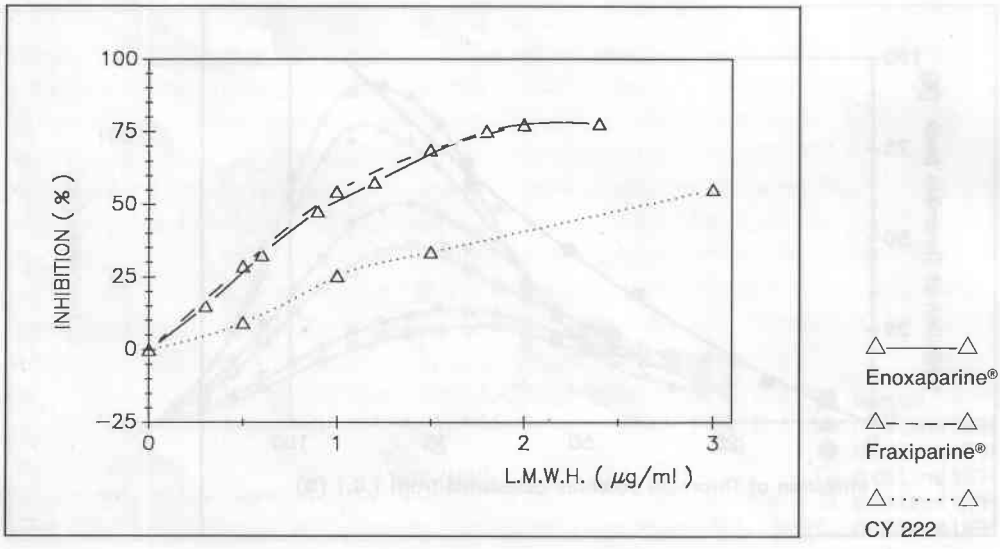
Fig. 5. Thrombin peak inhibition



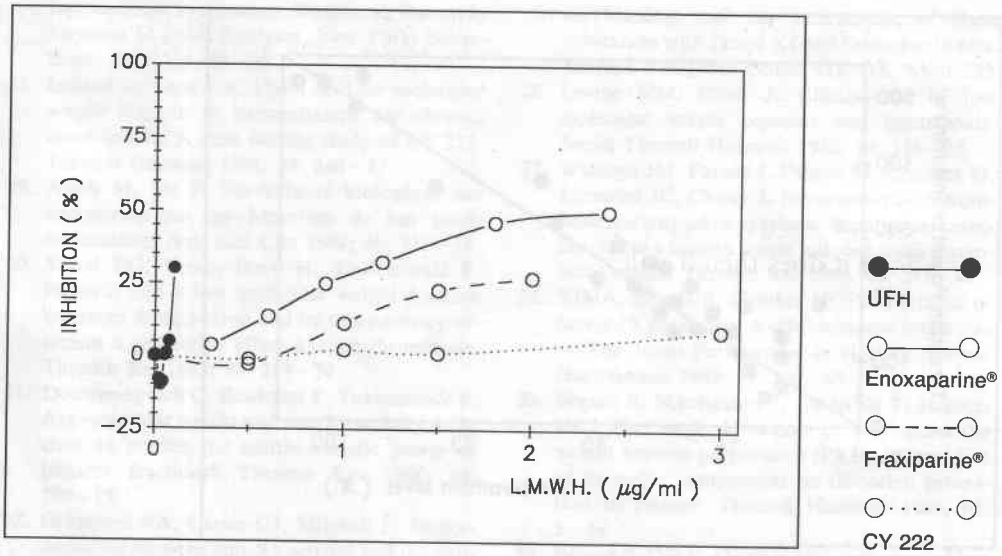



Fig. 6. Prothrombinase peak inhibition

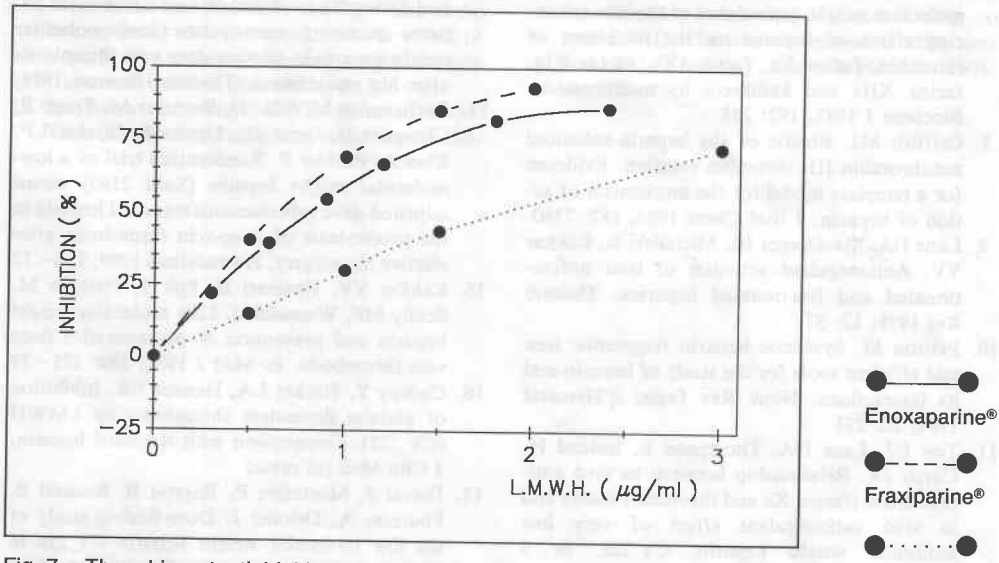



Fig. 7. Thrombin potential inhibition

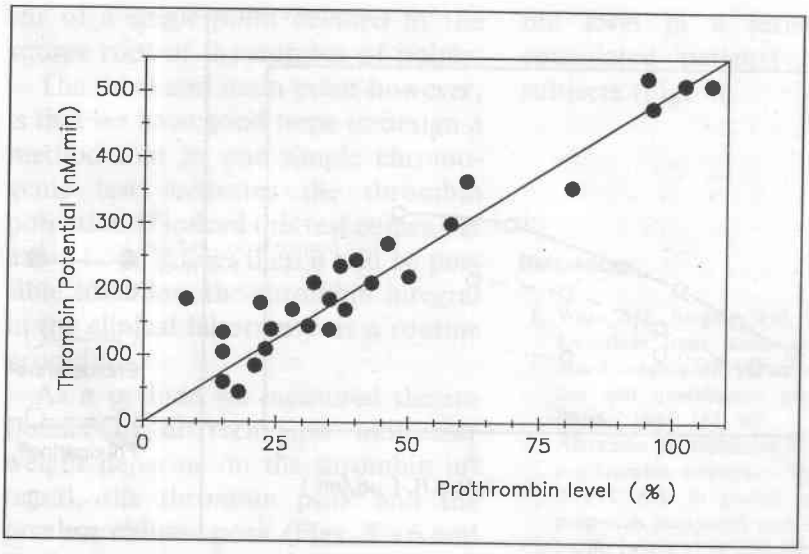



Fig. 8. The thrombin potential compared to the prothrombin level in anticoagulated patients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